# Chapter 18

# Deuterium Labeling for Neutron Structure–Function–Dynamics Analysis

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## Summary

Neutron scattering and diffraction provide detailed information on the structure and dynamics of biological materials across time and length scales that range from picoseconds to nanoseconds and from 1 to 10,000 Å, respectively. The particular sensitivity of neutrons to the isotopes of hydrogen makes selective deuterium labeling of biological systems an essential tool for maximizing the return from neutron scattering experiments. In neutron protein crystallography, the use of fully deuterated protein crystals improves the signal-to-noise ratio of the data by an order of magnitude and enhances the visibility of the molecular structure (Proc Natl Acad Sci U S A 97:3872–3877, 2000; Acta Crystallogr D Biol Crystallogr 61:1413–1417, 2005; Acta Crystallogr D Biol Crystallogr 61:539–544, 2005). In solution and surface scattering experiments, the incorporation of deuterium-labeled subunits or components into complex assemblies or structures makes it possible to deconvolute the scattering of the labeled and unlabeled subunits and to determine their relative dispositions within the complex (J Mol Biol 93:255–265, 1975). With multiple labeling patterns, it is also possible to reconstruct the locations of multiple subunits in ternary and higher-order complexes (Science 238:1403–1406, 1987; J Mol Biol 271:588–601, 1997; J Biol Chem 275:14432–14439, 2000; Biochemistry 42:7790–7800, 2003). In inelastic neutron scattering experiments, which probe hydrogen dynamics in biological materials, the application of site, residue, or region-specific hydrogen–deuterium-labeling patterns can be used to distinguish and highlight the specific dynamics within a system (Proc Natl Acad Sci U S A 95:4970–4975, 1998).

Partial, selective, or fully deuterated proteins can be readily produced by endogenous expression of recombinant proteins in bacterial systems that are adapted to growth in $D_2O$ solution and using selectively deuterated carbon sources. Adaptation can be achieved either by gradual step-wise increase in $D_2O$ concentration or, more directly, by plating cells on media of choice and selecting colonies that perform best for subsequent culture and inoculation. Scale-up growth and expression is typically performed in standard shaker flasks using either commercial or "home-grown" rich media (derived, for example, from cell lysates produced from algae grown in $D_2O$) or under more controlled conditions in defined minimal media. Cell growth is typically slower in deuterated media (>5 times slower) and yields are correspondingly lower. Once the target protein has been expressed, purification proceeds by the protocols developed for the hydrogenated protein. The deuteration levels of the final product are determined by mass spectrometry.

**Key words:** Protein, Membrane, Nucleic acid, Deuterium labeling, Cell culture, Over-expression, Deuterium exchange, Crystallography, Reflectometry, Small angle scattering, Contrast variation

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## 1. Introduction

Neutron scattering is exquisitely sensitive to the position, content, and dynamics of hydrogen atoms in materials and thus is a powerful tool for the characterization of structure-function and interfacial relationships in biological systems. Applications in biology range from the atomic resolution analysis of individual hydrogen atoms in enzymes through to meso- and macro-scale analysis of complex biological structures, membranes, and assemblies. Because neutrons interact with and scatter from nuclei, rather than with electrons, neutron scattering lengths ($b$) show little variation across the periodic table. Most importantly for biology, neutrons are extremely sensitive to hydrogen atoms and to the deuterium isotope, whose scattering length differs in both magnitude and phase, and selective substitution of hydrogen with deuterium can therefore be used to distinguish and highlight the position, structure, or dynamics of individual components within complex macromolecular systems or assemblies. Neutron studies are thus greatly enhanced by the design and production of specific, random, and uniform hydrogen/deuterium (H/D)-labeled biological macromolecules.

The degree and extent of deuterium labeling required for neutron scattering depends on the specific application. Neutron protein crystallography has the most stringent demands, requiring complete isotopic substitution of deuterium for hydrogen, which greatly reduces the hydrogen incoherent scattering background and significantly increase the signal-to-noise ratio of the diffraction data ***(1–3)***. In small-angle neutron scattering (SANS) applications, which rely on neutron contrast variation techniques, partial (~70–80%) deuterium labeling is generally sufficient to label, highlight, and map chemically distinct or D-labeled components of larger protein/protein or protein/lipid/nucleic acid complexes and assemblies ***(4–8)***. Similarly, neutron reflectometry using specific labeling and contrast variation allows the structure, composition, and organizational changes of membranes and of integral or membrane associated proteins to be dissected and examined in situ. In neutron spectroscopy, which accesses molecular dynamics in the nanosecond to picosecond range, more elegant amino acid residue, site, or regio-specific H-labeling of otherwise fully deuterated complexes can allow the internal dynamics of functional components to be analyzed in situ ***(9)***. Total, partial, or selective isotopic labeling is thus a powerful tool in neutron scattering analysis, which promises to provide new and more sophisticated ways to tackle complex problems in biology.

Here we describe approaches developed for the production of fully, partially, and selectively deuterated protein by endogenous expression of recombinant proteins in bacterial systems grown in $D_2O$ solution using deuterated carbon sources.

## 2. Materials

### 2.1. Deuterated Media Preparation

1. $D_2O$ (Cambridge Isotope Laboratories).
2. Minimal medium salts as shown in **Table 1**.
3. Trace metal solution as shown in **Table 1**.
4. Rotary Evaporator (Heidolph).
5. $D_8$-glycerol (Cambridge Isotope Laboratories).

### 2.2. Cell Adaptation

1. LB medium plate: 20 g/L LB powder (DIFCO, Lennox or Miller), 15 g/L Bacto-agar (DIFCO) autoclaved at 121°C for 15 min; antibiotic (1,000×) sterilized by filtration (*see* **Note 1**).
2. Minimal medium (2×): salt solution (2×), trace element solution (1,000×) as shown in **Table 1**. Trace element solution should be prepared fresh (*see* **Note 2**). The minimal medium is sterilized by filtration.
3. Hydrogenated minimal medium plate: 2× minimal medium sterilized by filtration; 2× warm (52°C) agar solution sterilized at 121°C for 15 min; 1,000× antibiotic solution sterilized by filtration (*see* **Note 3**).
4. Deuterated minimal medium (2×): 2× salt solution prepared in $D_2O$, 1,000× trace element solution prepared in $D_2O$ as shown in **Table 1** (*see* **Note 4**).

**Table 1**
**Minimal medium *(10)***

| Component | Initial concentration |
|---|---|
| $(NH_4)_2SO_4$ | 6.86 g/L |
| $KH_2PO_4$ | 1.56 g/L |
| $Na_2HPO_4 \cdot 2H_2O$ | 6.48 g/L |
| $(NH_4)_2$-H-citrate | 0.49 g/L |
| $MgSO_4 \cdot 7H_2O$ | 0.25 g/L |
| Trace metal solution | 1.0 mL/L |
| Glycerol | 5.0 g/L |
| *Trace metal solution*<br>0.5 g/L $CaCl_2 \cdot 2H_2O$, 16.7 g/L $FeCl_3 \cdot 6H_2O$, 0.18 g/L $ZnSO_4 \cdot 7H_2O$, 0.16 g/L $CuSO_4 \cdot 5H_2O$, 0.15 g/L $MnSO_4 \cdot 4H_2O$, 0.18 g/L $CoCl_2 \cdot 6H_2O$, 20.1 g/L EDTA | |

5. Deuterated minimal medium plate: 2× deuterated minimal medium sterilized by filtration; 2× warm (52°C) agar solution prepared in $D_2O$ sterilized at 121°C for 15 min; 1,000× antibiotic solution prepared in $D_2O$ sterilized by filtration (*see* **Note 3**).
6. 15-mL BD Falcon™ conical-bottom polypropylene tubes.
7. Vacuum-driven filtration and storage devices (Stericup Filter Units; Millipore).

### 2.3. Cell Culture

#### 2.3.1. Flask

1. 1× deuterated minimal medium supplemented with antibiotic.
2. Fernbach flask.
3. Induction solution: 1,000× IPTG prepared in $D_2O$.

#### 2.3.2. Bioreactor

1. 1× deuterated minimal medium supplemented with antibiotic.
2. Feeding solution: 10% deuterated glycerol, 0.2% $MgSO_4$ in $D_2O$ supplemented with antibiotic.
3. Base solution: 10% NaOD in $D_2O$.
4. Induction solution: 1,000× IPTG prepared in $D_2O$.
5. 1.25-L Bioflo 3000 Bioreactor (New Brunswick Scientific).
6. Polypropylene glycol (PPG) (Sigma-Aldrich).
7. Air, nitrogen (Air Liquide).
8. Storage bottle headpiece (Sartorius BBI Systems).
9. Dissolved oxygen (DO) probe (Broadley James).
10. pH probe (Broadley James).

### 2.4. Cell Lysis and Purification

1. Hydrogenated purification buffers (*see* **Note 5**).

### 2.5. Deuterium Back-Exchange

1. Final protein buffer prepared in $D_2O$ (*see* **Note 6**).
2. Centrifugal filter units (Amicon, Millipore).

## 3. Methods

This section describes a protocol for production of fully (per) deuterated protein. The levels of deuteration reached are greater than 95%. Substitution of the deuterated carbon source with a hydrogenated carbon source, and/or using $D_2O/H_2O$ mixed solutions to prepare the medium will produce lower levels of deuteration, which are sufficient for neutron contrast variation experiments such as SANS and reflectometry. Alternatively, using protocols originally developed for nuclear magnetic resonance (NMR) applications, (per)deuterated medium can be supplemented

with hydrogenated amino acids or their precursors for the preparation of selectively hydrogenated, deuterated proteins ***(11)***.

Culture growth in fully deuterated medium is typically slower than in hydrogenated media, and growth time can be can be long, especially when using a bioreactor. Therefore, plasmid loss over time can be a major problem in deuterated culture and can explain low or even null overexpression levels. The stability of the plasmid used requires careful consideration ***(12,13)***. An important parameter of plasmid stability is the selection marker. Under ampicillin selection, the β-lactamase protein that confers resistance is stored in the periplasmic space. An inner and an outer cell membrane limit the periplasmic space. "Leakiness" of the outer membrane leads to β-lactamase secretion into the culture medium. Selection then becomes rapidly ineffective because ampicillin can then be degraded by secreted β-lactamase. This can result in growth of bacteria that have lost their plasmids or contaminant cell growth. In contrast, the protein that confers kanamycin resistance is cytosolic and therefore less likely to leak into the culture medium. Expression vectors that confer ampicillin resistance to the host cell should therefore be avoided (*see* **Note 7**). The expression vector should also allow for overexpression induction in the last phase of the deuterated culture to avoid possible degradation of protein over time.

### *3.1. Preparation of Fully Deuterated Minimal Medium*

Production of fully (per)deuterated protein requires media prepared from 100% $D_2O$ and a perdeuterated carbon source. To prepare a "hydrogen-free" medium, special precautions need to be taken.

1. Dissolve hydrogenated and hydrated mineral salts in $D_2O$ so that labile hydrogen atoms are exchanged for deuterium and dry using a rotary evaporator. Repeat twice for a more complete exchange. The deuterated salts are then dissolved in $D_2O$ to make up the medium salt solution (*see* **Note 8**). A 2× salt solution can be prepared.
2. Similarly, hydrogenated and hydrated trace element salts should be dissolved in $D_2O$ to exchange hydrogen for deuterium and then dried using a rotary evaporator. Prepare a 1,000× solution and add to the salt solution.
3. Prepare a 1,000× antibiotic solution in $D_2O$ and add to the medium.
4. Any chemicals required for protein overexpression (substrate, cofactor, metal ion) can be dissolved in $D_2O$ and added to the medium. Again, these should be deuterated if possible.
5. Add $D_8$-glycerol and sterilize the medium by filtration using a vacuum-driven filtration and storage device (Stericup Filter Units; Millipore).

### *3.2. Cell Adaptation on Solid Medium*

Expression of (per)deuterated protein requires first an adaptation of the cells to growth in fully deuterated media. This adaptation can be made in a three-step process using solid media in standard

plates at 37°C. Adaptation can also be made in liquid media (*see* **Note 9**).

1. Plate freshly transformed cells on a hydrogenated solid LB medium plate.
2. Select a colony and plate on hydrogenated solid minimal medium.
3. After overnight growth, plate cells on fully deuterated (heavy water and deuterated carbon source) solid minimal medium. To prepare solid deuterated minimal medium plates, autoclave a 2× mixture of agar in $D_2O$. In parallel, prepare a 2× liquid deuterated minimal medium that has been supplemented with antibiotic and filter sterilized. Combine equal volumes of the warmed solutions and pour the plates. Once plated, cell growth on the deuterated plates is observed after 2–4 days of incubation.
4. Select a colony and transfer adapted cells to fully deuterated liquid medium. Once growth is established, fresh deuterated minimal medium can be inoculated in a 1:20 ratio. Cycling this step increases the initial growth rate.
5. At this point, large volume cultures can be inoculated. If required, adapted cells can be stored in 10-mL aliquots at −80°C after flash freezing in liquid nitrogen.
6. Thaw adapted cells stored at −80°C slowly on ice (*see* **Note 10**).
7. Before growing the actual inoculum, perform up to four transfers in freshly prepared deuterated media to refresh the cells, complete the adaptation, and improve the growth rates.
8. Prepare inoculum in standard sterilized and dried flasks in shaking incubators. The deuterated culture inoculum volume can be up to 1/10th of the starting culture volume, presuming that the inoculum is in the exponential growth phase and free of toxic byproduct (*see* **Note 11**). An $OD_{600}$ of 4 has been used successfully.

### 3.3. Culture

Deuterated cultures can be grown in flasks or, to reach higher cell density, in bioreactors. When using a bioreactor, the yield can be improved by first running a batch phase, followed by a fed-batch phase. Care should be taken to avoid any source of hydrogen (water drops, vapor) contamination.

#### 3.3.1. Flask

1. Sterilize and dry flasks.
2. Prepare deuterated minimal medium supplemented with antibiotic.
3. Inoculate the medium with a $D_2O$ adapted culture.
4. Shake at 180 rpm until the OD reaches 2.0.
5. Induce overexpression.
6. Stop the culture and harvest the cells when the expression level is satisfactory.

*3.3.2. Bioreactor*

1. Place and properly align the head plate on the 1.25-L vessel and tighten the screws.
2. Place the protective stainless steel cap over the bearing housing. Do not sterilize the rubber motor coupling.
3. Connect a 37-mm inlet filter (0.2 μm) to the sparger and a 50-mm exhaust filter (0.2 μm) to the condenser via a short length of tubing.
4. Attach tubing for the feed, base, and acid solutions to the appropriate ports. Wrap or clamp any open ends to maintain a sterile reactor (*see* **Note 12**). No solutions or probes should be added to the vessel before sterilization.
5. Prepare bottles with a storage bottle headpiece for feed, base, and acid solutions.
6. Autoclave the bioreactor and bottles at 121°C for 15 min.
7. Connect the water lines to the exhaust condenser and vessel jacket.
8. Turn on the water supply and then the bioreactor control unit.
9. Attach the inlet tubing to the inlet filter, remove the inlet clamp, and dry the vessel thoroughly with sterile-filtered, compressed air. Dry the feed, base, and acid bottles in a drying oven.
10. Check the tip of the DO probe for punctures or tears. Refill the tip with electrolyte solution if needed. Polarize the probe according to manufacturer's specifications (~6 h).
11. Calibrate the pH probe with standard solutions of known (pH 4 and 7).
12. Carefully sterilize the DO and pH probes with a 70% ethanol solution and insert them into the vessel.
13. Fill the vessel with deuterated medium through the inoculation port. Fill the feed, base, and acid bottles with solutions sterilized by filtration. Remove clamps and attach the bottles to the vessel using the corresponding tubing and pumps.
14. Calibrate the DO probe by sparging $N_2$ and air into the vessel to set the 0 and 100% calibrations, respectively.
15. Insert the temperature probe into the thermowell and set the bioreactor to the desired temperature.
16. Remove the protective stainless steel cover from the bearing housing and attach the rubber motor coupling. Attach the motor and connect it to the control unit.
17. Set the airflow to 0.5 L/min and the agitation rate to 200 rpm.
18. Inoculate the medium with a $D_2O$ adapted culture.
19. Begin controlling the pH and DO% with the bioreactor's control unit or PC-based control software. The initial DO% in the bioreactor vessel is 100% (no oxygen consumption) and the culture medium defines the pH. During the batch

phase, cell growth is not controlled and is close to the maximum growth rate. After inoculation, the DO% is automatically adjusted to 30% ***(14)*** by controlling the stirring rate (*see* **Note 13**). The pH, which generally changes as the carbon source is metabolized, is kept within 0.1 U of initial pH of the medium. This is controlled by automatic addition of base or acid solution (*see* **Note 14**).

20. Add 200 μL of PPG to prevent foam formation.
21. Occasionally sample and check the $OD_{600}$ of the culture during the growth (*see* **Note 15**).
22. During the fed-batch phase, provide the cells with fresh carbon source solution. The growth rate should be maintained constant but slightly lower than the maximum growth rate. This is performed by providing a limited amount of carbon source. A deuterated feeding solution is prepared with 10% deuterated glycerol, 0.2% $MgSO_4$, and antibiotic (*see* **Note 16**). The feeding rate can be determined manually by taking into consideration the regulation of the pH and of the DO%. The feeding rate is increased when a decrease in the base solution addition frequency and in the aeration is observed, both sign of depletion of the carbon source. The fed-batch phase can last up to 5 days. Alternatively, a control sequence can be used to automatically estimate appropriate feeding rates as the culture progresses. Expected yields are 1 g of cell paste per gram of carbon source used.
23. Protein overexpression can be induced at any time during the fed-batch phase because the cell growth rate is constant.
24. Stop the culture at the end of the induction period and harvest.

### 3.4. Cell Lysis and Purification

Standard protocols can be used for cell lysis using buffers and solutions prepared in $H_2O$ and hydrogenated media. Subsequent purification steps for the target protein can also be done in hydrogenated buffers and solutions following the protocols established for the "native" hydrogenated form of the protein. Although this allows labile deuterium atoms on amide or hydroxyl groups to exchange for hydrogen during the protein purification steps (accounting for ~20% of the deuterium content of typical proteins), these can be readily back-exchanged to deuterium by equilibration with a final deuteration buffer.

### 3.5. Deuteration Level

Mass spectrometry is used to calculate the deuteration level of the (per)deuterated protein. The theoretical molecular weight of (per) deuterated protein purified in hydrogenated buffer is given by $MW_{\text{partially deuterated}} = MW_{\text{hydrogenated}} + (\text{number of non-exchangeable deuterium}) \times 1.006$, where with 1.006 is the mass difference between deuterium and hydrogen. An example is given in **Table 2**. The deuteration level is then given by $(MW_{\text{partially deuterated}} - MW_{\text{hydrogenated}})_{\text{determined by mass spectroscopy}} / (MW_{\text{partially deuterated}} - MW_{\text{hydrogenated}})_{\text{theoretical}}$.

**Table 2**
**Theoretical molecular weight calculation of hydrogenated and (per)deuterated protein purified in hydrogenated buffer (all exchangeable deuterium atoms are considered to have exchanged to hydrogen during the purification). An example is given for rubredoxin, a 53-amino acid protein**

| AA | AA(H) MW | Number of AA | MW(H) | Non-exch. H/D | AA(H/D) MW | MW(H/D) |
|---|---|---|---|---|---|---|
| Ala | 71.0788 | 3 | 213.2364 | 4 | 75.1028 | 225.3084 |
| Arg | 156.1876 | 0 | 0 | 7 | 163.2296 | 0 |
| Asn | 114.1039 | 1 | 114.1039 | 3 | 117.1219 | 117.1219 |
| Asp | 115.0886 | 7 | 805.6202 | 3 | 118.1066 | 826.7462 |
| Cys | 103.1448 | 4 | 412.5792 | 3 | 106.1628 | 424.6512 |
| Glu | 129.1155 | 6 | 774.693 | 5 | 134.1455 | 804.873 |
| Gln | 128.1308 | 0 | 0 | 5 | 133.1608 | 0 |
| Gly | 57.052 | 5 | 285.26 | 2 | 59.064 | 295.32 |
| His | 137.1412 | 0 | 0 | 5 | 142.1712 | 0 |
| Ile | 113.1595 | 4 | 452.638 | 10 | 123.2195 | 492.878 |
| Leu | 113.1595 | 2 | 226.319 | 10 | 123.2195 | 246.439 |
| Lys | 128.1742 | 5 | 640.871 | 9 | 137.2282 | 686.141 |
| Met | 131.1986 | 0 | 0 | 8 | 139.2466 | 0 |
| Phe | 147.1766 | 2 | 294.3532 | 8 | 155.2246 | 310.4492 |
| Pro | 97.1167 | 5 | 485.5835 | 7 | 104.1587 | 520.7935 |
| Ser | 87.0782 | 2 | 174.1564 | 3 | 90.0962 | 180.1924 |
| Thr | 101.1051 | 1 | 101.1051 | 5 | 106.1351 | 106.1351 |
| Trp | 186.2133 | 2 | 372.4266 | 8 | 194.2613 | 388.5226 |
| Tyr | 163.176 | 2 | 326.352 | 7 | 170.218 | 340.436 |
| Val | 99 | 2 | 198 | 8 | 107.048 | 214.096 |
| End effect | 18 | 1 | 18 | | 18 | 18 |
| *Rubredoxin* | | 53 | 5,895.2975 | 120 | | 6,198.1035 |

*AA* amino acid; *AA(H) MW* hydrogenated amino acid molecular weight; *Number of AA* number of amino acid residues in the protein; *MW(H)* total contribution of amino acid to the protein molecular weight; *Non-exch. H/D* number of non-exchangeable hydrogen/deuterium in amino acid; *AA(H/D) MW* partially deuterated amino acid molecular weight; *MW(H/D)* total contribution of amino acid to the partially deuterated protein molecular weight

### 3.6. Deuterium Back-Exchange

Labile deuterium atoms exchange to hydrogen during the purification process and need to be exchanged back to deuterium. Back exchange to deuterium is completed by three dilution–concentration cycles of the protein in deuterated buffer using a centrifugal filter unit with the appropriate molecular weight cutoff (Amicon, Millipore). An overnight break before the last cycle may favor back-exchange of buried and protected hydrogen atoms (*see* **Note 17**).

### 3.7. Site-Specific Hydrogenation of Deuterated Proteins

Selectively methylated, triple-labeled proteins have been previously prepared for NMR applications. In those methods, the ketoacid precursor, [3-$^{2}$H] $^{13}$C α-ketoisovalerate, was used ***(11)***. By simply changing the isotopic composition of the α-ketoisovalerate precursor and the minimal medium, ($^{1}$H-δ methyl)-leucine and ($^{1}$H-γ methyl)-valine can be selectively incorporated into an otherwise deuterated protein for neutron protein crystallography or spectroscopy applications using the method below.

1. Sterilize and dry flasks.
2. Prepare [3-$^{2}$H] α-ketoisovalerate from unlabeled α-ketoisovalerate by warming a 25 mM solution of α-ketoisovalerate in $D_2O$ at pH 12.5, 45°C for 3 h ***(11)***.
3. Prepare fully deuterated minimal medium using 0.3%, w/v D-glucose (1,2,3,4,5,6,6,-D7, 98%) from Cambridge Isotope Laboratories supplemented with antibiotic.
4. Inoculate the medium with a $D_2O$-adapted culture and incubate at 37°C with 250-rpm shaking.
5. Approximately 1 h before induction, add 100 mg of [3-$^{2}$H] α-ketoisovalerate/L culture.
6. Induce overexpression with 1 mM IPTG at an $OD_{600}$ of 0.9.
7. Harvest cells after 4 h of induction.

## 4. Notes

1. Antibiotic is added when the medium cools below 52°C.
2. Metalloprotein overexpression experiments may require the addition, subtraction, or substitution of certain salts in the trace element solution.
3. Add sterile-filtered minimal medium to warm autoclaved agar solution while stirring to avoid agar lumps.
4. Ensure that all of the glassware used is free of any trace of water (drops, vapor).
5. Although no significant modifications are expected compared with purifying hydrogenated material, a small-scale preparation

is recommended to check that the affinity of the deuterated material for the resins is not altered by isotopic effects. pH is the parameter most likely affected if differences in affinity are observed.

6. pH and pD differ by ~0.4 U in 100% $D_2O$. The relation between the pH read on a pH meter ($pH_{measured}$) and the pD of a solution is given by $pD = pH_{measured} + 0.4$.
7. Antibiotic resistance can be switched using the Ez-Tn5 Kan-2e insertion kit (Epicentre Biotechnologies).
8. $MgSO_4$ must be dissolved last or precipitation will be observed in the medium.
9. Cells can be adapted using liquid media exclusively, starting from liquid LB, to liquid hydrogenated minimal medium and to liquid deuterated minimal medium. This may require increasing stepwise the $D_2O$ concentration of the liquid deuterated medium (e.g., 10, 50, 80, and 100%). This alternate protocol requires the cells to be transferred when in exponential growth phase—or adaptation may fail.
10. The inoculum can be started from freshly adapted cells.
11. The pH of the inoculum should be around the pH of the medium. A large pH shift indicates the presence of growth by-products that may eventually be toxic as they accumulate.
12. The exhaust tubing should be covered with aluminum foil and not clamped.
13. Depending on the starting optical density, the consumption of the $O_2$ initially present can range from several minutes to several hours.
14. The pH decreases when glycerol is used as carbon source. An increase of pH can be used as an indicator for increasing the feeding rate.
15. The $OD_{600}$ can be continuously monitored if a probe is available.
16. Cost of the deuterated carbon source should be considered when preparing the feeding solution. A higher concentration can be used if cost is not an issue.
17. Dialysis can also be used, but the required volume of deuterated buffer is larger.

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